UNIVERSITI PUTRA MALAYSIA

PHENOTYPIC AND MOLECULAR CHARACTERIZATIONS OF *STREPTOCOCCUS* SPP. ISOLATED FROM BOVINE MAMMARY GLANDS

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By

MD. FIROZ MIAN

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Veterinary Medicine Universiti Putra Malaysia

October 2001

DEDICATION

TO THE MEMORY OF MY BEREAVED FATHER AND TO MY MOTHER
TO MY WIFE MASUDA AND DAUGHTER FARHIN
TO MY BROTHER GULAM MUSTAFA

AND

TO LATE Dr. M. FAZLUR RAHMAN, CHIEF SCIENTIFIC OFFICER, BLRI

Abstract of the thesis presented to the Senate of the Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PHENOTYPIC AND MOLECULAR CHARACTERIZATIONS OF *STREPTOCOCCUS SPP.* ISOLATED FROM BOVINE MAMMARY GLANDS

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Chairman: Abdul Rahim Mutalib, DVM, MS, Ph.D.

Faculty: Veterinary Medicine

Sixty-two streptococcal isolates comprising 20 *Streptococcus agalactiae*, 18 *S. dysgalactiae* and 24 *S. uberis* isolates were recovered from clinical and subclinical cases of bovine mastitis from different dairy herds in the Selangor state in Malaysia. A simple biochemical test scheme formulated on the basis of seven biochemical reactions allowed the identification of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* isolates within 24 hours. *Streptococcus agalactiae* isolates were β-haemolytic, CAMP positive, utilized hippurate, salicin and raffinose; *S. dysgalactiae* isolates were α-haemolytic and fermented only trehalose and raffinose, while *S. uberis* isolates showed positive reactions to esculin, inulin and mannitol. The API 20 Strep System characterized accurately 100% of *S. agalactiae* and *S. dysgalactiae* isolates, and 96.1% of *S. uberis* isolates although some variable reactions among the isolates within the species were observed. Majority of the isolates were susceptible to most of the antimicrobial agents tested. All *S. agalactiae*, *S. dysgalactiae* and *S. uberis* isolates were susceptible to oxacillin and nitrofurantoin, while 30% of the isolates were resistant to tetracycline. Most of the *S. dysgalactiae* isolates showed resistance

to kanamycin and cephalexin, and *S. agalactiae* isolates to knamycin. *Streptococcus dysgalactiae* isolates showed higher level of resistance compared to *S. agalactiae* and *S. uberis*. Serotyping of the streptococcal isolates using monospecific antisera in agar gel double immunodiffusion revealed that all *S. agalactiae* isolates were typeable and demonstrated the type patterns II (60%), Ia (20%), III (15%) and IV (5%). On the other hand, 17 (94.4%) *S. dysgalactiae* and 22 (91.6%) *S. uberis* isolates were also identified.

The SDS-PAGE and Western-blotting analyses revealed antigenic heterogeneity among the isolates of the bovine *Streptococcus* species examined. Sodium dodecylsulphate polyacrylamide gel electrophoresis could differentiate the three streptococcal species on the basis of their characteristic polypeptide bands. The Western blot analysis also revealed obvious differences in immunogenic proteins between the streptococcal species. Moreover, isolates within each species produced variable protein bands on PAGE analysis and variable immunogenic proteins by Western blotting which let the basis to group them into distinct PAGE and immunoblot fingerprint profiles respectively.

Random amplified polymorphic DNA (RAPD) analysis was evaluated for its capacity to distinguish strains within the species of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* and for epidemiological subtyping. Three single primers were used for each species to generate characteristic RAPD fingerprints. The DNA fingerprint patterns obtained with each primer were distinct and reproducible. The RAPD fingerprints generated could be useful in delineating the strains of *S. agalactiae*, *S. dysgalactiae*

and *S. uberis.* The intraspecies typing efficiency was significantly improved by the parallel use of three primers. The RAPD results showed high level of genetic diversity within strains of the streptococcal species.

The amplification of the DNA encoding 16S rRNA genes by polymerase chain reaction with single set of primers complementary to 16S rRNA gene regions generated characteristic single amplicon that enabled identification and differentiation of the three streptococcal species. The restriction fragment length polymorphism (RFLP) analysis of the amplified 16S rRNA gene regions with the restriction enzymes *Msp*I and *Rsa*I produced reproducible fingerprint patterns indicating high level of genetic diversity among the isolates of the streptococcal species. Higher heterogeneity was observed within *S. uberis* and *S. dysgalactiae* isolates than the *S. agalactiae* isolates. The discriminatory powers of the two enzymes were to some extent similar.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN FENOTIP DAN MOLEKUL *STREPTOCOCCUS SPP.* YANG DIPENCIL DARIPADA KELENJAR MAMA BOVIN

Oleh

MD. FIROZ MIAN

Oktober 2001

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Enam puluh dua pencilan streptokokus terdiri daripada 20 *Streptococcus agalactiae*, 18 *S. dysgalactiae* dan 24 *S. uberis* telah diperolehi daripada kes klinikal dan subklinikal mastitis bovin berbagai kelompok lembu tenusu dalam negeri Selangor, Malaysia. Suatu skema ujian biokimia mudah yang dirumus berasaskan tujuh tindak balas, telah membolehkan pengenalpastian pencilan *S. agalactiae*, *S. dysgalactiae*, dan *S. uberis* dalam tempoh 24 jam. Pencilan *Streptococcus agalactiae* ialah α-hemolisis, positif pada ujian CAMP, pengguna hipurat, salisin dan rafinosa; pencilan *S. dysgalactiae* ialah α-hemolisis dan hanya menapai trehalosa dan rafinosa, sambil *S. uberis* menunjukkan tindak balas positif terhadap eskulin, inulin, dan manitol. API 20 Strep System mencirikan dengan tepat 100% daripada pencilan S. *agalactiae* dan *S. dysgalactiae*, 96.1% daripada pencilan *S. uberi*, walaupun terdapat pelbagai tindak balas di kalangan pencilan dalam sesuatu spesies. Sebahagian besar daripada pencilan ini rentan terhadap kebanyakan agen antimikrob yang diuji.

Kesemua pencilan *S. agalactiae, S. dysgalactiae* dan *S. uberis* adalah rentan terhadap oksasilin dan nitrofurantoin, sambil 30% daripada pencilan tahan tetrasiklin. Kebanyakan daripada pencilan *S. dysgalactiae* menunjukkan ketahanan terhadap kanamisin dan sefaleksin, dan pencilan *S. agalactiae* pula terhadap kanamisin. Pencilan *Streptococcus dysgalactiae* menunjukkan ketahanan lebih tinggi berbanding *S. agalactiae* atau *S. uberis*. Penserotipan pencilan streptokokus mengguna antiserum monokhusus dalam gel agar-agar pengimunoresapan dedua menunjukkan pencilan *S. agalactiae* boleh ditip dan menyatakan pola tip II (60%), III (20%), dan IV (5%). Disebaliknya, 17 (94,4%) pencilan *S. dysgalactiae* dan 22 (91.6%) pencilan *S. uberis* telah dikenal pasti.

Analisis SDS-PAGE dan pensapan Western menunjukkan keheterogenan antigen di kalangan pencilan spesies *Streptococcus* bovin yang dikaji. Elektroforesis gel poliakrilamida natrium dodesilsulfat boleh membeza tiga spesies streptokokus berasaskan jalur polipeptida cirian. Analisis sap Western juga menunjukkan kelainan nyata dalam protein imunogen di antara spesies streptokokus. Tambahan pula pencilan di dalam setiap spesies menghasilkan jalur protein pelbagai pada analisis PAGE dan protein imunogen pelbagai pada pengsapan Western, yang mengsahihkan asas untuk mengumpulkan pencilan ini masing-masing kepada profil sidikjari PAGE dan imunosap.

Analisis DNA polimorfik terkuat rawak (RAPD) telah dinilaikan untuk keupayaannya membeza strain di kalangan spesies *S. agalactiae, S. dysgalactiae* dan *S. uberis* dan untuk pengsubtipan epidemiologi. Pola sidikjari DNA yang diperolehi daripada setiap primer adalah jelas dan boleh dihasil semula. Sidikjari RAPD yang

dijanakan mungkin berguna dalam membeza di antara strain *S. agalactiae*, *S. dysgalactiae* dan *S. uberis*. Kecekapan pengetipan intraspesies nyata lebih baik dengan penggunaan tiga primer. Hasil RAPD menunjukkan yang aras kepelbagaian genetik di kalangan strain spesies streptokokus adalah tinggi.

Penguatan gen 16S rRNA pengekod DNA melalui tindak balas rangkaian polimerase mengguna satu set primer pelengkap kepada kawasan gen 16S RNA menjanakan amplikon tunggal cirian yang membolehkan untuk pengenalpastian dan pembezaan tiga spesies streptokokus dilakukan. Analisis polimorfosime panjang fragmen pengehadan (RFLP) terhadap kawasan gen 16S RNA terkuat dengan mengguna enzim pengehadan *MspI* dan *RsaI* menghasilkan pola sidikjari boleh dihasil semula, menunjukkan yang adanya kepelbagaian genetik aras tinggi di kalangan pencilan spesies streptokokus. Keheterogenan lebih tinggi telah dicerap di kalangan pencilan *S. uberis* dan *S. dysgalactiae* berbanding pencilan *S. agalactiae*. Kuasa penmbezaan untuk dua enzim ini agak sama.

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I certify that an Examination Committee met on 13th October 2001 to conduct the final examination of Md. Firoz Mian on his Doctor of Philosophy thesis entitled "Phenotypic and Molecular Characterizations of *Streptococcus spp.* Isolated from Bovine Mammary Glands" in accordance with Universiti Pertanian Malaysia (Higher degree) Act 1980 and Universitti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy.

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I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at Universiti Putra Malaysia or other institutions.

MD. FIROZ MIAN

Date: 01- 11- 2001

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab	Antibody
AP-PCR	Arbitrary Primed-Polymerase Chain Reaction
bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celcius
cfu	Colony forming unit
cm	Centimeter
CMT	California Mastitis Test
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleotide triphosphate
Dr.	Doctor
e.g.	For example
EDTA	Ethelene Diamine tetra-acetate
ELISA	Enzyme Linked Immunosorbent Assay
g	Gram
g/l	Gram per litre
G+C	Guanine + Cytosine
H_2O_2	Hydrogen peroxide
h/hrs	Hour/Hours
HIS	Hyperimmune Serum
i.e.	That is
i.m.	Intramuscular
IgA	Immunoblobulin A
IgG	Immunoglobulin G
Kbp	Killobase pairs
kDa	Killodalton
M	Molar
Mab	Monoclonal Antibody
M.W.	Molecular Weight
Mg	Milligram
min/mins	Minute/Minutes
ml	Millilitre
mM	Millimolar
nm	Nanometer
O.D.	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline Tween 20
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentartion
PFGE	Pulsed Field Gel Electrophoresis